

430 Rec'd PCT/PT 27 JAN 2000

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(REV 12-29-99)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

GJE-35

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

**09/463549**

INTERNATIONAL APPLICATION NO.

PCT/GB98/02214 ✓

INTERNATIONAL FILING DATE

July 24, 1998 ✓

PRIORITY DATE CLAIMED

July 28, 1997; and ✓

December 22, 1997

TITLE OF INVENTION

Nucleic Acid Sequence Analysis ✓

APPLICANT(S) FOR DO/EO/US

Daniel Henry Densham ✓

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:  
Submission of Sequence Listing Under §§1.821-1.825  
Amendment Under 37 CFR §1.825(a) Through (c)  
Sequence Listing on paper and computer readable format containing  
the same information

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U.S. APPLICATION NO. (if known) **09/463549**INTERNATIONAL APPLICATION NO.  
PCT/GB98/02214ATTORNEY'S DOCKET NUMBER  
GJE-3517. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

Neither international preliminary examination fee (37 CFR 1.482)  
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO ..... \$970.00

International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO ..... \$840.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but  
international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$690.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$670.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$96.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =****CALCULATIONS** PTO USE ONLY

\$ 840.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30  
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	35 - 20 =	15	X \$18.00
Independent claims	4 - 3 =	1	X \$78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00

\$ 270.00

\$ 78.00

\$ 0.00

**TOTAL OF ABOVE CALCULATIONS =**

\$ 1,188.00

Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement  
must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

\$

**SUBTOTAL =**

\$ 1,188.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30  
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

**TOTAL NATIONAL FEE =**

\$ 1,188.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

\$

**TOTAL FEES ENCLOSED =**

\$ 1,188.00

Amount to be  
refunded:

\$

charged:

\$

a. ☐ A check in the amount of \$\_\_\_\_\_ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-0065 in the amount of \$\_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
overpayment to Deposit Account No. 19-0065. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

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SIGNATURE.

Doran R. Pace

NAME

38,261

REGISTRATION NUMBER

09/463549

January 27, 2000

AMENDMENT UNDER 37 CFR  
§1.825(a) THROUGH (c)  
Patent Application  
Docket No. GJE-35

428 Rec'd PCT/PTO 27 JAN 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Daniel Henry Densham  
Docket No. : GJE-35  
For : Nucleic Acid Sequence Analysis

Box PCT  
Assistant Commissioner for Patents  
Washington, D.C. 20231

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)

Sir:

Please amend the above-identified application as follows, in order to comply with the requirements of 37 CFR §§1.821-1.825:

In the Specification

Please insert the accompanying Sequence Listing as new page 16.

In the Claims

Please renumber pages 16-18 as 17-19.

Remarks

This amendment is made to conform the application with the provisions of 37 CFR §§1.821 through 1.825. I hereby certify that no new material is being added by this submission.

Respectfully submitted,



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DRP/sl

Attachment: New page 16 of subject application.

NUCLEIC ACID SEQUENCE ANALYSISField of the Invention

This invention relates to a method for determining the sequence of a polynucleotide.

5 Background of the Invention

10 The ability to determine the sequence of a polynucleotide is of great scientific importance. For example, the Human Genome Project is an ambitious international effort to map and sequence the three billion basis of DNA encoded in the human genome. When complete, the resulting sequence database will be a tool of unparalleled power for biomedical research. The major obstacle to the successful completion of this project concerns the technology used in the sequencing process.

15 The principal method in general use for large-scale DNA sequencing is the chain termination method. This method was first developed by Sanger and Coulson (Sanger et al. Proc. Natl. Acad. Sci. USA 1977; 74: 5463-5467), and relies on the use of dideoxy derivatives of the four  
20 nucleoside triphosphates which are incorporated into the nascent polynucleotide chain in a polymerase reaction. Upon incorporation, the dideoxy derivatives terminate the polymerase reaction and the products are then separated by gel electrophoresis and analysed to reveal the position at  
25 which the particular dideoxy derivative was incorporated into the chain.

Although this method is widely used and produces reliable results, it is recognised that it is slow, labour-intensive and expensive.

30 An alternative sequencing method is proposed in EP-A-0471732, which uses spectroscopic means to detect the incorporation of a nucleotide into a nascent polynucleotide strand complementary to a target. The method relies on an immobilised complex of template and primer, which is  
35 exposed to a flow containing only one of the different nucleotides. Spectroscopic techniques are then used to measure a time-dependent signal arising from the polymerase

catalysed growth of the template copy. The spectroscopic techniques described are surface plasmon resonance (SPR) spectroscopy, which measures changes in an analyte within an evanescent wave field, and fluorescence measuring techniques. However, limitations of this method are recognised; the most serious for the SPR technique being that, as the size of the copy strand grows, the absolute size of the signal also grows due to the movement of the strand out of the evanescent wave field, making it harder to detect increments. The fluorescence measuring techniques have the disadvantage of increasing background interference from the fluorophores incorporated on the growing nascent polynucleotide chain. As the chain grows, the background "noise" increases and the time required to detect each nucleotide incorporation needs to be increased. This severely restricts the use of the method for sequencing large polynucleotides.

There is therefore a need for an improved method for determining the sequence of polynucleotides which significantly increases the rate at which a polynucleotide is sequenced and which is preferably carried out by an automated process, reducing the complexity and cost associated with existing methods.

#### Summary of the Invention

The present invention is based on the realisation that the measurement of electromagnetic or other radiation can be used to detect a conformational and/or mass change in a polymerase enzyme which occurs when a nucleotide is incorporated into a nascent polynucleotide strand.

According to the present invention, a method for sequencing a polynucleotide comprises the steps of:

(i) reacting a target polynucleotide with a polymerase enzyme immobilised on a solid support, and the different nucleotides, under conditions sufficient for the polymerase reaction; and

(ii) detecting the incorporation of a specific nucleotide complementary to the target polynucleotide, by measuring radiation.

The radiation may be applied to a sample using a number of techniques, including surface-sensitive detection techniques, where a change in the optical response at a solid optical surface is used to indicate a binding interaction at the surface. In a preferred embodiment of the invention, the technique used is evanescent wave spectroscopy, in particular surface plasmon resonance (SPR) spectroscopy.

In an embodiment of the invention, the nucleotides used in the method include a blocking group at the 3' position, and optionally at the 5' position, which prevents incorporation of the nucleotides into the polynucleotide strand. However, the blocking groups may be selectively removed to allow incorporation to occur. By using the blocked nucleotides, it is possible for the method to be carried out using all the nucleotides present in the reaction at any one time. The selective removal of the blocking groups is carried out in a way that ensures the detection of each incorporated nucleotide. The method may therefore proceed on a "real-time" basis, to achieve a high rate of sequence analysis.

#### 25 Description of the Drawings

The invention will be described by way of example only with reference to the following drawings, where:

Figure 1 is a schematic illustration of polynucleotide sequence analysis using SPR spectroscopy; and

30 Figure 2 illustrates the different response signals detected for the polymerisation of each of the different nucleotides.

Figure 3 illustrates the synthesis procedure for the double blocked nucleotides.

#### 35 Description of the Invention

The present method for sequencing a polynucleotide involves the analysis of the kinetic interaction between a

polymerase enzyme, a target polynucleotide and a complementary nucleotide. Measurement of the kinetic interaction is carried out by monitoring the changes in or absorption of electromagnetic or other radiation that occurs if the reaction proceeds.

The term "polynucleotide" as used herein is to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other hybridising nucleic acid-like molecules, e.g. peptide nucleic acid (PNA).

Typically, the method is carried out by applying electromagnetic radiation, by using the techniques of surface plasmon resonance or nuclear magnetic resonance. However, other techniques which measure changes in radiation may be considered, for example spectroscopy by total internal reflectance fluorescence (TIRF), attenuated total reflection (ATR), frustrated total reflection (FTR), Brewster angle reflectometry, scattered total internal reflection (STIR) or evanescent wave ellipsometry.

Techniques other than those requiring electromagnetic radiation are also envisaged, in particular photochemical techniques such as chemiluminescence, and gravimetric techniques including resonant systems such as surface acoustic wave (SAW) techniques and quartz crystal microbalance (QCM) techniques.

Surface plasmon resonance (SPR) spectroscopy is a preferred method, and measures the properties of a solution by detecting the differences in refractive index between the bulk phase of the solution and the evanescent wave region. Incident monochromatic light is reflected at a specific angle off a solid optical (sensor chip) surface on the opposite side to a sample under study. The light extends into the sample for a very short distance and is affected by an interaction at the surface.

Suitable sensor chips are known in the art. Typically, they comprise an optically transparent material, e.g. glass, and a thin reflective film, e.g. silver or gold. For a review of SPR spectroscopy see European Patent

Publication No. 0648328 (the entire disclosure of which is incorporated herein by reference).

5 Nuclear magnetic resonance (NMR) spectroscopy is another preferred method, and measures the magnetic properties of compounds. Nuclei of compounds are energetically orientated by a combination of applied magnetic field and radio-frequency radiation. When the energy exerted on a nucleus equals the energy difference between spin states (the difference between orientation  
10 parallel or anti-parallel to the direction of the applied fields), a condition known as resonance is achieved. The absorption and subsequent emission of energy associated with the change from one spin state to the other, is detected by a radio-frequency receiver.

15 An important aspect of the method of the present invention is the use of a polymerase enzyme immobilised onto a solid support. Immobilisation of the polymerase offers several important advantages for the success of this method. Firstly, the problem of random "noise" associated with measuring energy absorption in soluble molecules is  
20 reduced considerably. Secondly, the problem of noise from the interaction of any substrate (e.g. nucleotides) not directly involved with the polymerase is reduced, as the polymerase can be maintained within a specifically defined area relative to the field of measurement. This is  
25 particularly relevant if the technique used to measure the changes in radiation requires the measurement of fluorescence, as in TIRF, where background fluorescence increases as the nascent chain grows. Also, if SPR spectroscopy is used, the polymerase reactions are  
30 maintained within the evanescent wave field and so accurate measurements can be made irrespective of the size of the polynucleotide. Finally, as neither the target polynucleotide nor the oligonucleotide primer is  
35 irreversibly attached to the solid surface, it is relatively simple to regenerate the surface, to allow



further sequencing reactions to take place using the same immobilised polymerase.

Immobilisation may be carried out using standard procedures known in the art. In particular, immobilisation using standard amine coupling procedures may be used, with attachment of ligand-associated amines to, say, a dextran or N-hydroxysuccinimide ester-activated surface. In a preferred embodiment of the invention, the polymerase is immobilised onto a SPR sensor chip surface which maintains the polymerase in close proximity to the sensor surface where changes in the refractive index may be measured. Examples of procedures used to immobilise biomolecules to optical sensors are disclosed in EP-A-0589867, and Löfas et al., Biosens. Bioelectron. (1995) 10:813-822.

The polymerase used in the invention may be of any known type. For example, the polymerase may be any DNA-dependent DNA polymerase. If the target polynucleotide is a RNA molecule, then the polymerase may be a RNA-dependent DNA polymerase, i.e. reverse transcriptase, or a RNA-dependent RNA polymerase, i.e. RNA replicase. In a preferred embodiment of the invention, the polymerase is Taq polymerase. In a further preferred embodiment of the invention, the polymerase is either *E. coli* polymerase III holoenzyme (McHenry, Ann. Rev. Biochem. 1988; 57:519), T7 polymerase (Schwager et al., Methods in Molecular and Cellular Biology (1989/90); Vol.1(4):155-159, or Bacteriophage T7 gene 5 polymerase complexed with *E. coli* Thioredoxin (Tabor et al., J. Biol. Chem. (1987); 262:1612-1623). Each of these polymerase enzymes allows a binding with the target polynucleotide to occur with high fidelity and therefore maintains a polymerase-polynucleotide complex, even when polymerisation is not actively taking place.

The polymerase III holoenzyme is composed of three subassemblies that function to create the processive enzyme: (I) the polymerase core, including the polymerase subunit  $\alpha$ ; (II)  $\beta$ -dimer subunit which acts as a bracelet-

like structure around DNA; and (III) a subassembly of two subunits,  $\tau$  and  $\gamma$ , used to bind and hydrolyse ATP to form the  $\beta$ -dimer around the DNA.

As a first step in the sequencing process, the target  
5 polynucleotide may be brought into contact with an appropriate primer in hybridising/polymerisation buffer. Typically, the buffer will be at a sufficiently high temperature to disrupt (or melt) any secondary structures that exist on the target polynucleotide. On cooling, the  
10 primer will anneal to its complement on the target. This sample may then be brought into contact with the immobilised polymerase, to form the target polynucleotide/polymerase complex.

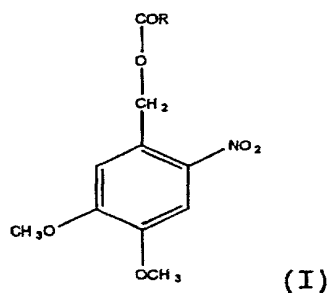
In one embodiment of the invention, the addition of  
15 the nucleotides is controlled so that the different nucleotides are added sequentially to the polymerase/target complex. For example, dGTP may be added and allowed to flow over the polymerase/polynucleotide complex; any incorporation is then detected. Unbound dGTP flows out of  
20 the reaction site and a further nucleotide is introduced. In this manner, the detection of a kinetic interaction can be correlated to the particular nucleotide present at that time and the polynucleotide sequence can therefore be determined.

25 The method may also be carried out with all the different nucleotides present. For this to be carried out successfully, it is necessary for the nucleotides to incorporate a blocking group at least at the 3' position, but preferably at the 3' and 5' positions. The blocking  
30 groups may be light-sensitive and can be removed by applying light of a defined wave length, to release the active molecule. If the nucleotides incorporate blocking groups at both the 3' and 5' positions, the blocking groups should be capable of being distinguished on the basis of  
35 their spectral absorbancy, i.e. it should be possible to remove selectively one of the blocking groups by applying a specific wavelength of light which does not remove the

other blocking group. It is also desirable that the blocking group at the 3' position requires the light to be applied for a longer duration than that required to remove the blocking group at the 5' position. This allows the blocking groups to be distinguished by both spectral and temporal means.

Generally, the light-sensitive blocking groups undergo photolysis at wavelengths in the range from 200nm to 450nm. The blocking groups will typically be derived from a compound of the formula  $R^1-[O-CO-]X$  wherein  $R^1$  is a photolabile group and X is a leaving group. For example,  $R^1$  is o-nitrobenzyl. Particularly preferred blocking groups include the o-nitrobenzyl protecting groups described in WO-A-92/10092 and WO-A-97/39151. These groups include nitroveratryloxycarbonyl (NVOC), nitropiperonyloxycarbonyl (NPOC),  $\alpha$ -methyl-nitroveratryloxycarbonyl (MeNVOC),  $\alpha$ -methyl-nitropiperonyloxycarbonyl (MeNPOC) and 1-pyrenylmethyloxycarbonyl (PYMOC).

A suitable 3' blocking group is a (4,5-dimethoxy-2-nitrobenzyl)oxycarbonyl group which can be formed by reaction of the nucleotide with a compound of formula (I):

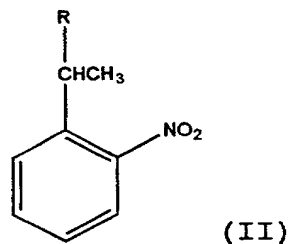


wherein R is any suitable esterifying group, e.g. methyl. This blocking group can be selectively removed by a pulse of light with a wavelength of 360 nm.

A suitable blocking group at the 5' position is 1-(2-nitrophenyl)ethyl group (II):

5

10



15 wherein R is any suitable functional group, e.g. halogen. This blocking group may be selectively removed at a wavelength of 260 nm.

By way of example, double-blocked nucleotides are injected over the primed target polynucleotide (held in association with a high fidelity polymerase complex), and monochromatic light is focussed upstream of the polymerase at a wavelength sufficient to release the blocking group from the terminal phosphate of each nucleotide. The nucleotides are then able to flow over the bound polymerase, and incorporation into the nascent polynucleotide strand can occur. However, as the blocking group at the 3' position remains bound, only one nucleotide is incorporated. A measurement of the kinetic interaction will therefore provide information as to the particular nucleotide incorporated into the nascent chain. The polymerase used may be a high fidelity polymerase which does not dissociate readily from the target when the reaction stops. Alternatively, a competitive inhibitor may be used to prevent the polymerase dissociating from the target.

After measuring the incorporated nucleotide, a pulse of monochromatic light is focused on the blocking group

within the polymerase catalytic site, to remove the blocking group at the 3' position. The monochromatic light may be pulsed for a longer duration than that required for removal at the 5' position, and so only the blocking group associated with the nucleotide in the polymerase complex will undergo removal. This reduces the likelihood of the addition of nucleotides not associated with the polymerase complex.

Once the 3' blocking group is released, the polymerase reaction is allowed to continue as further nucleotides arrive at the polymerase reaction site. Uncontrolled polymerisation is prevented by alternating the pulses of light required to remove the blocking groups.

While it is preferred to use the double-blocked nucleotides, as described above, the procedure may also be carried out using nucleotides having a blocking group at the 3' position only. In this case, it is desirable to use a competitive inhibitor of the polymerase, which will reduce the probability of a nucleotide lacking a blocking group at the 3' position being incorporated into the nascent chain. A suitable competitive inhibitor of polymerase is carbonyldiphosphonate (COMDP).

The following Example illustrates the invention with reference to the drawings.

#### Example

The following analysis was carried out on a modified BIAcore 2000 system (BIAcore AB, UPPSALA, Sweden) with a sensor chip CM5 (Research grade, BIAcore AB) as the optical sensor surface. The instrument was provided with an integrated  $\mu$ m-fluidic cartridge (IFC) which allows analysis in four cells by a single sample injection.

#### Preparation of polymerase

*E. Coli* polymerase III holoenzyme was prepared according to (Millard et al., Methods Enzymol. (1995); 262:22) using hydrophobic interaction chromatography on valyl-Sepharose, to purify the holoenzyme at high salt concentrations. After purification, the hollow enzyme was

concentrated using the ion-filtration technique described by Kirkegaard et al, Anal. Biochem. (1972); 50:122.

#### Immobilisation of the Polymerase

Immobilisation of the polymerase to the sensor chip surface was carried out according to (Jönsson et al., Biotechniques (1991); 11:620-627). Briefly, the sensor chip environment was equilibrated with Hepes buffer (10 mM Hepes, 150 mM NaCl, 0.05% surfactant P20(BIAcore AB, Uppsala, Sweden), pH 7.4). Equal volumes of N-hydroxysuccinimide (0.1 M in water) and N-ethyl-n'-(dimethylaminopropyl) carbodiimide (EDC) (0.1 M in water) were mixed together and injected across the chip (CM5) surface, to activate the carboxymethylated dextran. The polymerase III Subassembly core (160  $\mu$ l, 500 U) was mixed with 10 mM sodium acetate (100  $\mu$ l, pH 5) and injected across the activated surface. Finally, residual N-hydroxysuccinimide esters on the sensor chip surface were reacted with ethanolamine (35  $\mu$ l, 1 M in water, pH 8.5), and non-bound polymerase was washed from the surface. The immobilisation procedure was performed with a continuous flow of Hepes buffer (5  $\mu$ l/min) at a temperature of 25°C.

#### Oligonucleotides

Two oligonucleotides were synthesised using standard phosphoramidite chemistry. The oligonucleotide defined as SEQ ID No. 1 was used as the target polynucleotide, and the oligonucleotide defined as SEQ ID No. 2 was used as the primer.

CAAGGAGAGGACGCTGTCTGTCTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG

30

SEQ ID No. 1

CTCTCCCTTCTCTCGTC

SEQ ID No. 2

The two oligonucleotides were reacted under hybridising conditions to form the target-primer complex.

35

The primed DNA was then suspended in buffer (20 mM Tris-HCl, pH 7.5, 8 mM MgCl<sub>2</sub>, 4% (v/v) glycerol, 5 mM

dithiothreitol (DDT), 40  $\mu$ g bovine serum albumin) containing 21  $\mu$ g ssDNA binding-protein and the DNA pol III sub-assembly, required to form the bracelet-like structure (1.6 pmol  $\beta$ -dimer and 195 fmol  $\gamma$  subunits). 0.5 mM ATP was present together with 60  $\mu$ M carbonyldiphosphonate (COMDP). In this reaction, the  $\gamma$  subunit acts as a molecular matchmaker, hydrolysing ATP to place the  $\beta$ -dimer subunits onto the DNA to form the polymerase sub-assembly (Studwell et al, UCLA Symp. Mol. Cell. Biol. New Ser. 1990; 127: 153).

The primed DNA/sub-assembly complex was then injected over the polymerase III on the sensor chip surface at a flow-rate of 5  $\mu$ m/min, and allowed to bind to the polymerase via the action of the  $\gamma$  subunits.

In this experiment, magnesium and ATP are needed for the Pol III to bind to the primed DNA. However, magnesium also promotes removal of the primer by the proof-reading 3' - 5' exonuclease activity. This problem is circumvented by including the carbonyldiphosphonate, which is a competitive inhibitor of polymerase activity (a pol III lacking the 3'-5' exonuclease activity may be used to avoid this particular problem).

A continuous flow of 60  $\mu$ M carbonyldiphosphonate was maintained over the chip surface, to prevent the exonuclease activity from removing the primer from the target DNA.

#### Nucleotides Incorporating Two Blocking Groups

Each nucleotide (dCTP, dTTP, dGTP and dATP) contained a 1-(2-nitrophenyl)ethyl blocking group at the 5' position, and a (4,5-dimethoxy-2-nitrobenzyl) oxycarbonyl blocking group at the 3' position as shown in Figure 3. The synthesis of the double blocked nucleotides was as follows:  
**Stage 1:- Synthesis of (4,5-dimethoxy-nitrobenzyl)oxycarbonyl-nucleoside triphosphate.**

The same overall method was applied to dGTP, dCTP and dTTP. A mixture of dATP dihydrate (0.4mmol) and approximately 3mmol of 4,5-dimethoxy-2-

nitrophenyldiazomethane, freshly prepared from 900mg (4 mmol) of 4,5-dimethoxy-2-nitrophenylhydrazine (synthesized by treatment of 6-nitroveraldehyde with hydrazine monohydrate in chloroform by the procedure of Wootton and  
5 Trentham, Photochemical Probes in Biochemistry (Nielsen, P.E., Ed,) NATO ASI Ser. C, Vol. 272, p277-296 (1989), was stirred in 15 ml of DMSO at room temperature in the dark for 40 h. Monitoring of the reaction by TLC in a chloroform/methanol (5:1 v/v) solvent system revealed the  
10 appearance of a spot with Rf 0.54 corresponding to the caged nucleotide. DMSO, unreacted diazo compound, and reaction products with low polarity were removed by repetitive extraction with 60 ml of ether. The residual material, which, among other substances, contained  
15 unreacted nucleotide and the desired product, was dissolved in a minimal amount of chloroform and separated by flash chromatography on a silica column (3 x 30 cm). Elution using 100% chloroform and methanol/chloroform (95:5 v/v) removed the hydrophobic side products of 4,5-dimethoxy-2-nitrophenyldiazomethane from the column. The fractions  
20 were dried on a rotary evaporator. 78 mg of the caged product was then lyophilised. The overall yield was 45%. The 3' blocked 4,5-dimethoxy-2-nitrobenzyl oxycarbonyl dATP was isolated directly with higher purity by  
25 preparative reverse-phased HPLC from the crude product.

**Stage 2:- Addition of the 5' 1-(2-nitrophenyl)ethyl group to the 3' 4,5-dimethoxy-2-nitrobenzyl oxycarbonyl blocked dATP.**

A mixture of 4,5-dimethoxy-2-nitrobenzyl oxycarbonyl  
30 5' dATP (0.4mmol) and approximately 3mmol of 1-(2-nitrophenyl)diazoethane, freshly prepared from 716.7 mg (4mmol) of hydrazone of 2-nitroacetophenone (synthesized by treatment of 2-nitroacetophenone with hydrazine monohydrate in ethanol) and 2.9g (30mmol) of MnO<sub>2</sub> (90%) in 20 ml of  
35 chloroform by the procedure of walker et al (walker et al, Methods Enzymol. 1989; 172:288-301), was stirred in 15 ml of DMSO at room temperature in the dark for 40 h.



Monitoring the reaction by TLC in a chloroform/methanol (5:1 v/v) solvent system revealed the appearance of a pair of spots with  $R_f$  0.68 and  $R_f$  0.58, corresponding to the two diastereoisomers of the axial and the two diastereoisomers of the equatorial form of the 1(2-nitrophenyl)ethyl ester of 4,5-dimethoxy-2-nitrobenzyl oxycarbonyl 5' dATP, respectively. DMSO, unreacted diazo compound, and reaction products with low polarity were removed by repetitive extraction with 50 ml ether.

The residual material, which contained among other substances unreacted 4,5-dimethoxy-2-nitrobenzyl oxycarbonyl 5' dATP and the desired double blocked dATP, was dissolved in a minimal amount of chloroform and separated by flash chromatography on a silica column 3 x 30 cm. Elution using 100% chloroform removed hydrophobic side products of 1-(2-nitrophenyl)diazoethane from the column. The product was dried on a rotary evaporator. Lyophilization gave 74 mg of the caged compound. The overall yield was 57%.

0.2 mM of each nucleotide was present in the polymerisation buffer (1 mM Tris-HCl pH 8.8, 5 mM KCl, 0.15 mM  $MgCl_2$ , 0.01 % (w/v) gelatin).

#### DNA Sequencing

Figure 1 shows a SPR sensing system and fluidic cell (7), having a means for applying electromagnetic radiation (1) to a sensor chip (2) with an immobilised polymerase enzyme (3) at the sensor surface, an inlet (4) for introducing the different nucleotides into the cell and two focusing assemblies (5) and (6) for pulsing monochromatic light into the cell.

The different nucleotides are introduced into the fluidic cell (7) at a flow rate of 30  $\mu$ l/min., at a temperature of 25°C and a data collection rate of 10 Hz. As the nucleotides pass the focusing assembly (5), monochromatic light at a wavelength of 260 nm is pulsed to remove the blocking group at the 5' position. The nucleotides then flow over the sensor chip (2) and contact

the target polynucleotide/ polymerase complex (3) which is held in place by the  $\beta$ -dimer sub-assembly. Since the 3' position on the primer sequence is free to react, polymerisation may take place as a nucleotide is incorporated onto its complement on the target polynucleotide. This incorporation is then detected by the monochromatic p-polarised light of the SPR device. No further polymerisation occurs, since the incorporated nucleotide has a blocking group at the 3' position.

10 Monochromatic light of wavelength 360 nm is then pulsed by the focusing assembly (6) at the site of polymerisation. The high flow rate in the fluidic cell ensures that nucleotides not bound to the polymerase are removed from the cell before sufficient energy has been absorbed to release their 3' blocking groups.

15

Once the 3' blocking group has been released from the polymerised nucleotide, further polymerisation may occur.

Figure 2 shows the results from the sequencing experiment with each nucleotide incorporated into the nascent chain being detected. The results show a sequence complementary to that of SEQ ID No. 1.

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CLAIMS

1. A method for sequencing a polynucleotide, comprising the steps of:

- 5 (i) reacting a target polynucleotide with a polymerase enzyme immobilised on a solid support, and the different nucleotides, under conditions sufficient for the polymerase reaction; and
- 10 (ii) detecting an effect consequent on the incorporation of a specific nucleotide complementary to the target polynucleotide.

2. A method according to claim 1, wherein the effect in step (ii) is detected by measuring radiation.

15 3. A method according to claim 1 or claim 2, wherein steps (i) and (ii) are conducted with each of the different nucleotides in turn, until incorporation is detected, and then repeated.

4. A method according to claim 1 or claim 2, wherein step (i) is conducted with all the nucleotides present.

20 5. A method according to any preceding claim, wherein the nucleotides comprise a 3' blocking group which is removed after the polymerase reaction.

25 6. A method according to claim 5, wherein the blocking group can be selectively removed by pulsed monochromatic light.

30 7. A method according to claim 5 or claim 6, wherein the nucleotides comprise a further blocking group at the terminal phosphate group of the triphosphate chain, and the further blocking group is removed prior to the removal of the 3' blocking group.

8. A method according to claim 7, wherein the further blocking group can be selectively removed by pulsed monochromatic light under conditions different from those required to remove the 3' blocking group.

35 9. A method according to claim 8, wherein the further blocking group is removed by pulsing the monochromatic

light for a duration different from that required to remove the 3' blocking group.

10. A method according to any preceding claim, wherein step (i) further comprises introducing a competitive  
5 inhibitor of the polymerase enzyme.

11. A method according to any preceding claim, wherein the target polynucleotide of step (i) is bound to the polymerase enzyme by a  $\beta_2$  dimer complex.

12. A method according to any preceding claim, wherein the  
10 polymerase is *E. coli* DNA polymerase III or T7 polymerase.

13. A method according to any of claims 1 to 11, wherein the polymerase is Taq polymerase.

14. A method according to any of claims 1 to 11, wherein the polymerase is reverse transcriptase.

15. 15. A method according to any preceding claim, wherein step (ii) comprises detection of a change in resonance signal over time.

16. A method according to any preceding claim, wherein the radiation is electromagnetic.

20 17. A method according to claim 16, wherein the electromagnetic radiation is in the infra-red spectrum.

18. A method according to any preceding claim, wherein step (ii) comprises using surface plasmon resonance.

25 19. A method according to claim 16, wherein the electromagnetic radiation is in the radio-frequency spectrum.

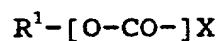
20. A method according to claim 19, wherein the incorporation of a nucleotide is detected using NMR.

30 21. A method according to any preceding claim, wherein the polynucleotide is DNA.

22. A sensor chip comprising a polymerase enzyme immobilised thereon.

23. A nucleotide comprising a blocking group at the 3' position and at the terminal phosphate group of the triphosphate chain, wherein the two blocking groups are  
35 removable by monochromatic light of different wavelengths.

24. A nucleotide according to claim 23, wherein the blocking groups are derived from a compound of the formula



5

wherein  $R^1$  is a photolabile group and X is a leaving group.

25. A nucleotide according to claim 23 or claim 24, wherein the blocking group at the 3' position is an o-nitrobenzyloxycarbonyl group.

10 26. A nucleotide according to any of claims 23 to 25, wherein the blocking group at the terminal phosphate is an o-nitrobenzyl group.

27. A nucleotide according to any of claims 23 to 26, wherein the blocking group at the 3' position is a (4,5-dimethoxy-2-nitrobenzyl)oxycarbonyl group.

15 28. A nucleotide according to any of claims 23 to 27, wherein the blocking group at the terminal phosphate is a 1-(2-nitrophenyl)ethyl group.

29. An apparatus for sequencing a polynucleotide, comprising an optical sensor chip, a light source, an imaging device and a photodetector, wherein the sensor chip is as defined in claim 22.

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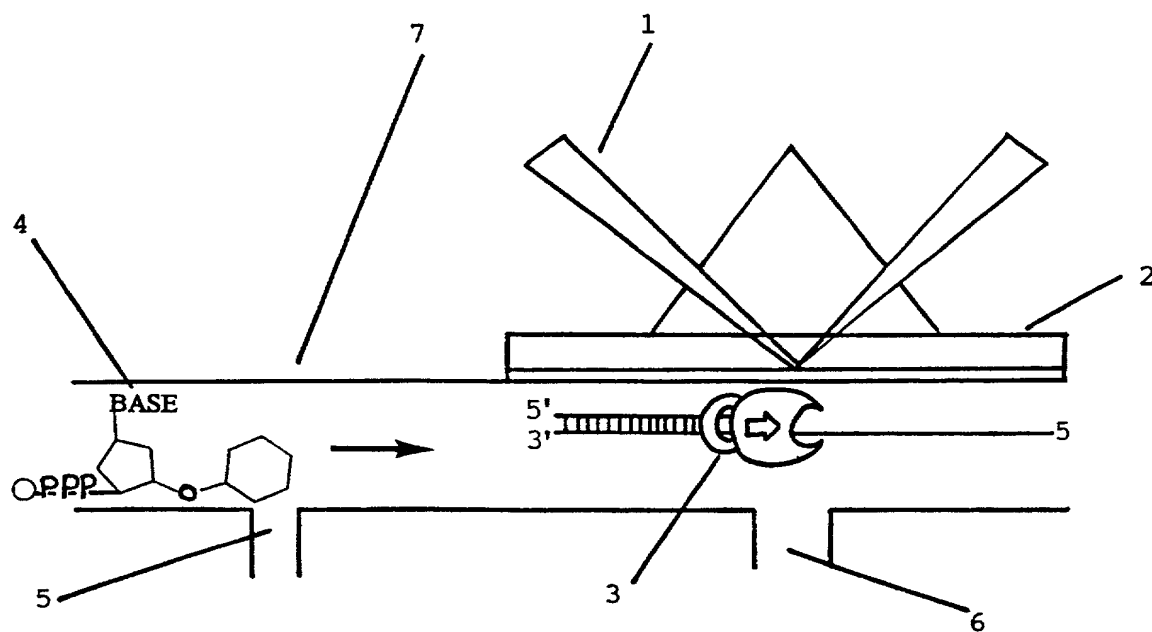


Fig. 1

2/5

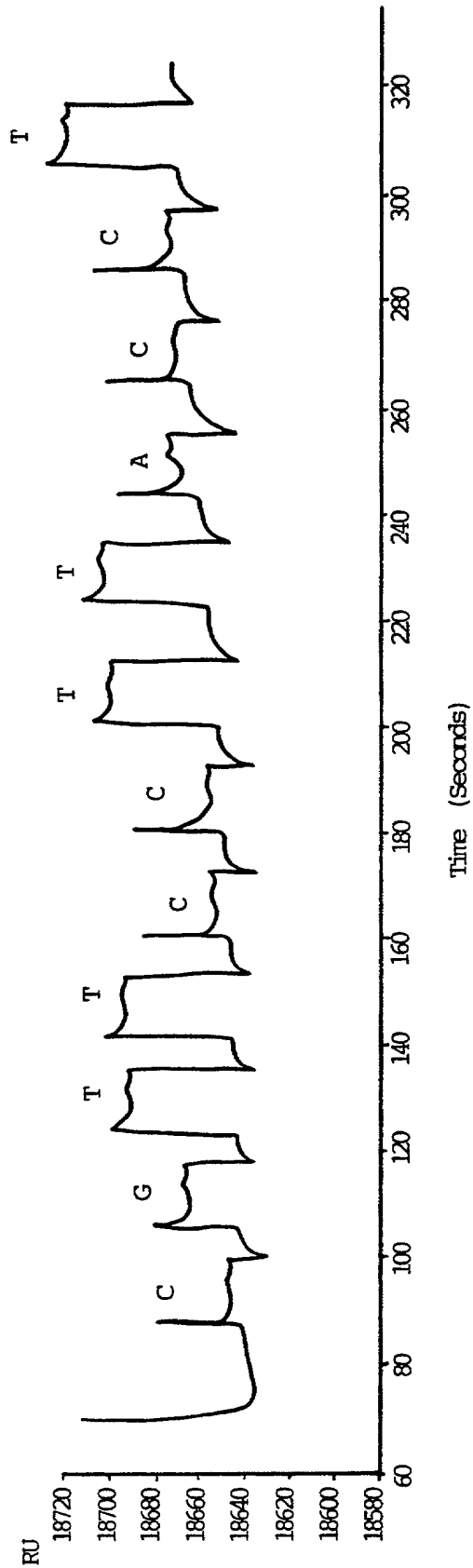


Fig. 2a

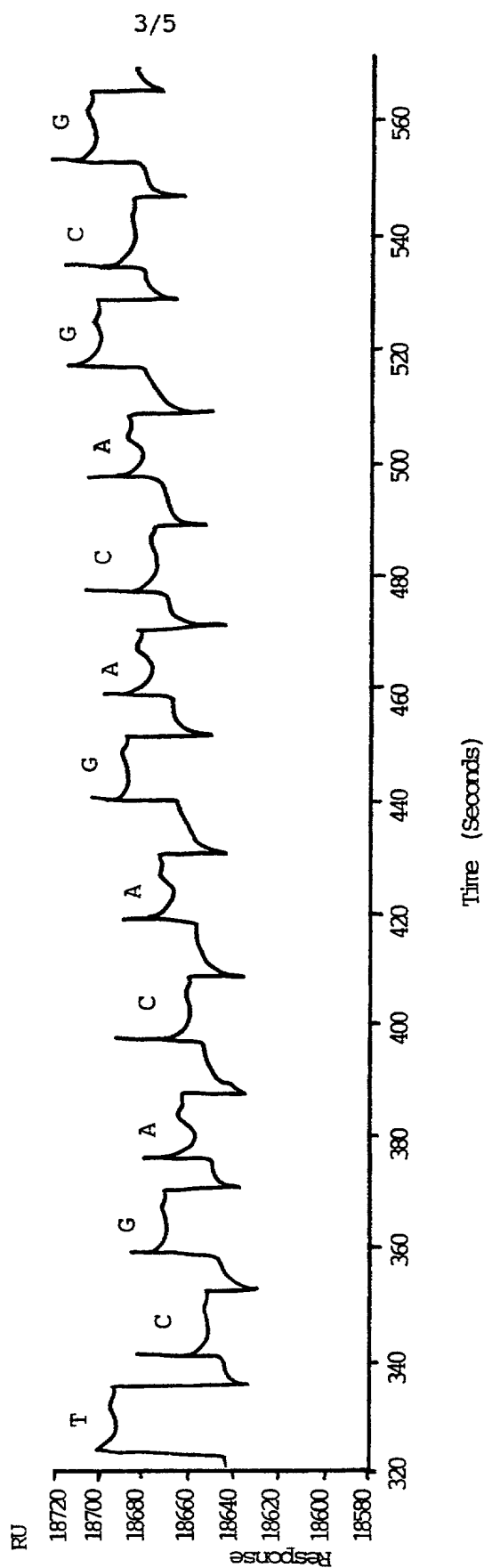


Fig. 2b



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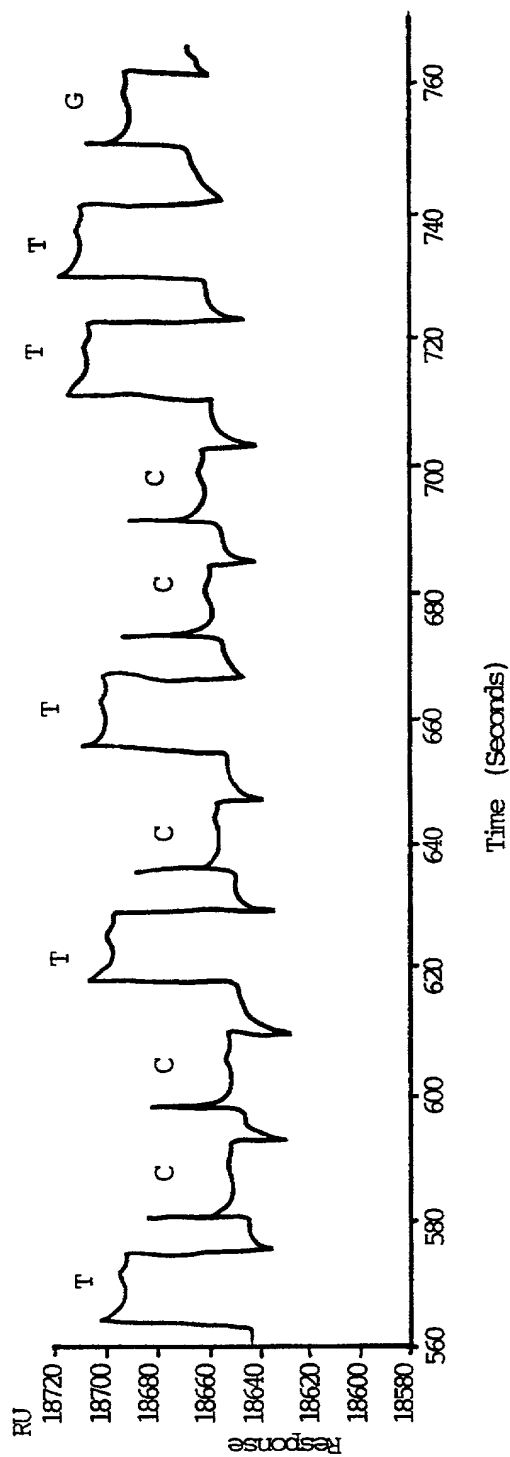


Fig. 2c

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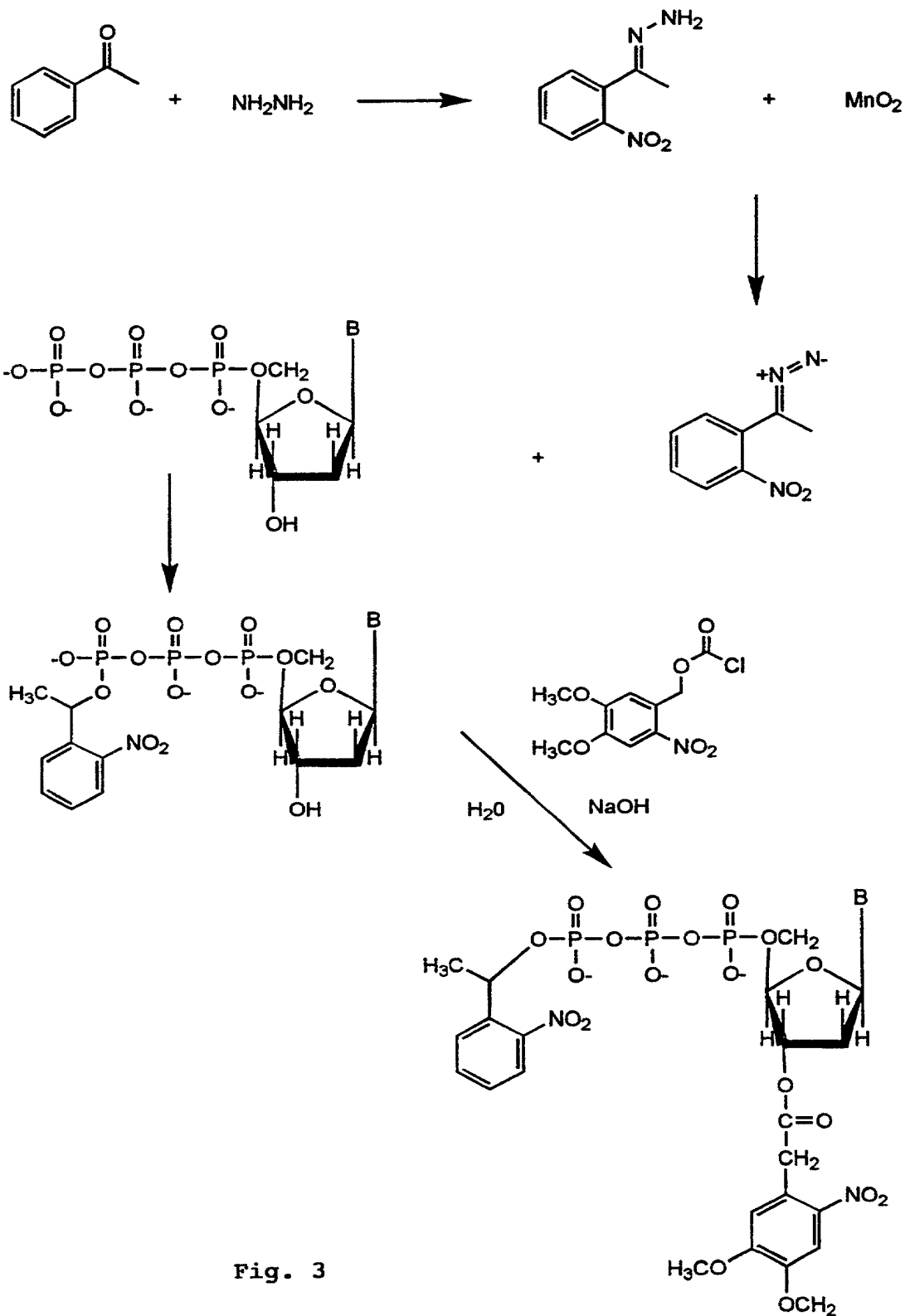


Fig. 3

## DECLARATION AND POWER OF ATTORNEY

USA  
GJE-35

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of subject matter which is claimed and for which a patent is sought on an invention entitled  
**NUCLEIC ACID SEQUENCE ANALYSIS**

the specification of which ☐ is attached hereto or

☒ was filed on 24 JUL 1998 as United States Application Number or PCT International Application Number PCT/GB98/02214 and was amended on (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for a patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
9715942.0	GB	28 JUL 1997 ✓	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
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As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: David R. Saliwanchik, Reg. 31,794; Jeff Lloyd, Reg. 35,589; Doran R. Pace, Reg. 38,261; Christine Q. McLeod, Reg. 36,213; Jay M. Sanders, Reg. 39,355; James S. Parker, Reg. 40,119; Jean E. Kyle, Reg. 36,987 and Timothy H. Van Dyke, Reg. P-43,218.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C 1001 and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

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Date of signature

05/12/99

## SEQUENCE LISTING

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